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Original Article

Association of *RASSF1* expression with radiosensitivity/prognosis in esophageal squamous cell carcinoma

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Abstract: To ascertain if ras association domain family 1 (RASSF1) expression in esophageal squamous cell carcinoma (ESCC) can predict response to radiotherapy. The expression of RASSF1 was measured by immunohistochemical staining prospectively in 76 patients with ESCC who underwent radiotherapy between July 2008 and December 2011. The association between RASSF1 expression and clinicopathological variables and treatment response was assessed by the $\chi^{(2)}$ test. The Kaplan-Meier curves were used to estimate probabilities of overall survival (OS) and progression-free survival (PFS). Cox proportional hazards regression model was used to independent predictors. $P<0.05$ was considered significant. Patients were followed-up for median months 18 (range: 4-61). Positive expression of RASSF1 manifested as brown staining in the cytoplasm or cell membranes, and significantly decreased in 46 of 76 (60.5%) of ESCC tissues compared with the matched normal tissues 95.0% (1/20) (P=0.003). Positive RASSF1 expression displayed a significant correlation with lower clinical stage (P=0.035), a better response to RT (P=0.001), and improved PFS (P=0.000) and OS (P=0.000). Multivariate analyses revealed that *RASSF1*-positive expression (Hazard ratio: 3.387, 95% confidence interval: 1.964-5.841, P=0.000) and complete response (Hazard ratio: 4.741, 95% confidence interval: 2.683-8.378, P=0.000) were significant prognostic factors. Therefore, RASSF1-positive expression is involved in the sensitization of ESCC to radiotherapy and the prognosis of patients with ESCC.

Keywords: Esophageal squamous cell carcinoma, radiotherapy, ras association domain family 1 (*RASSF1*), prognosis

Introduction

Esophageal cancer is the eighth most common cancer and sixth most common cause of cancer-related death, with 400,156 deaths reported worldwide in 2012 [1]. In China, esophageal squamous cell carcinoma (ESCC) accounts for 95% of all esophageal cancers [2]. Despite incorporation of new therapeutic approaches, it remains an aggressive disease with a dismal prognosis [3].

Radiation is a curative treatment option for patients with locally advanced ESCC who are not candidates for surgery. Unfortunately, persistent or recurrent disease has been reported in 46-68% of patients [4].

"Radiosensitization" is thought to be a key factor that affects therapeutic efficacy. However, biomarkers for radiosensitization are lacking. Identification of biomarkers for prediction of radiosensitization could provide useful indicators for individualized radiotherapy of patients with esophageal cancer.

Most scholars agree that multiple genetic regulatory mechanisms, particularly activation of oncogenes and inactivation of tumor-suppressor genes, underlie the occurrence and progression of cancer [5-8]. RAS is a family of related proteins expressed ubiquitously in all cell lineages and organs. The tumor suppressor-gene *RAS association domain family gene 1* (*RASSF1*) was identified in human chromosome 3p21.3 in 1982 [9]. The *RASSF1* gene comprises eight exons alternatively spliced to produce eight isoforms (*RASSF1*A-H) that have distinct functional domains, including the Ras associa-

tion (RA) domain [10, 11]. The *RASSF1* gene encodes RAS effective protein, which participates in RAS-related cellular signaling pathways and regulates oncogenesis, as well as the differentiation, proliferation, and apoptosis of multiple malignant tumor cells [12].

Studies have revealed increased expression of RASSF1 in normal human tissues, but lower levels in some tumor cells (e.g., lymphoma, pulmonary carcinoma, and melanoma), suggesting a relationship between reduced expression of the *RASSF1* gene and tumor oncogenesis [13]. In the present study, we focused on the tumor-suppressor gene *RASSF1*, which has a critical modulatory function in cellular interactions within healthy human bodies.

In the present study, we analyzed *RASSF1* expression in ESCC. We investigated the relationship between protein expression of *RASSF1* and clinicopathologic significance and prognosis in ESCC patients who underwent radiotherapy.

Patients and methods

Patient selection

The study was approved by the Ethical Committee of the Anyang Cancer Hospital [no. 2014(6)]. Written informed consent was obtained from each patient before using their tissues for this study, and the results of the study would not be communicated to the participants.

All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Between July 2008 and December 2011, 76 patients who underwent radiotherapy for ESCC in the Department of Radiation Oncology in the Anyang Cancer Hospital (Henan Province, China) were selected retrospectively. Patients with technically unresectable cancer, patients who refused to undergo surgery, or those considered medically unfit for surgery were eligible for definitive radiotherapy.

Inclusion criteria were histological proof of ESCC and no neoadjuvant therapy. Exclusion criteria were history of other types of cancer, double primary lesions, or death during radiotherapy.

The study cohort comprised 42 males and 34 females (mean age, 62 (range, 46-84; median, 70) years. All patients were questioned thoroughly regarding their medical history and had laboratory examinations (complete blood count, serum biochemistry). Also, they underwent a physical examination, esophagography, upper gastrointestinal endoscopy (with or without ultrasound), CT of the chest and abdomen and, if necessary, ^{18}F-fluorodeoxyglucose-positron emission tomography. This battery of tests was undertaken to ascertain ESCC stage.

Histopathology and tumor, node, metastasis (TNM) staging were carried out by two pathologists (X. M. Li and J. L. Li) based on criteria set by the Union for International Cancer Control in 2002 [14]. Twenty-six patients were stage I-II, and 50 subjects were stage III-IV. Six cases were T1, 35 were T2, 31 were T3, and 4 were T4. Five subjects were grade I, 57 were grade II, and 14 were grade III. Fifty-five patients also had lymph node (LN) metastasis, and 21 did not. Twenty normal esophageal tissues were studied as controls.

Radiotherapy

All patients received extended elective nodal irradiation and were treated with 59.4-64 (median, 59.4) Gy delivered at 1.8-2 Gy per fraction over 6-7 weeks. Treatment was delivered by linear accelerators with 6-MV photons. Treatment planning was based 3D-conformal radiotherapy or intensity-modulated radiotherapy. Further radiotherapy boosts were administered to cervical and supraclavicular LNs depending on LN size.

Immunohistochemical (IHC) staining

Biopsy specimens were obtained *via* pretreatment endoscopy with one sample for each patient. Paraffin-embedded blocks were sectioned at a thickness of 3 μm. Tissue sections were dewaxed with xylene and rehydrated through a series of ethanol gradients. Then, antigen retrieval and blocking was undertaken with rabbit immunosera. Sections were incubated with antibodies rabbit monoclonal against *RASSF1* (1:100 dilution; Abcam, Cambridge, UK) at 37°C for 1 h. After thorough washing with phosphate-buffered saline (PBS) on a shaker, tissue sections were incubated with a secondary antibody for 30 min, washed

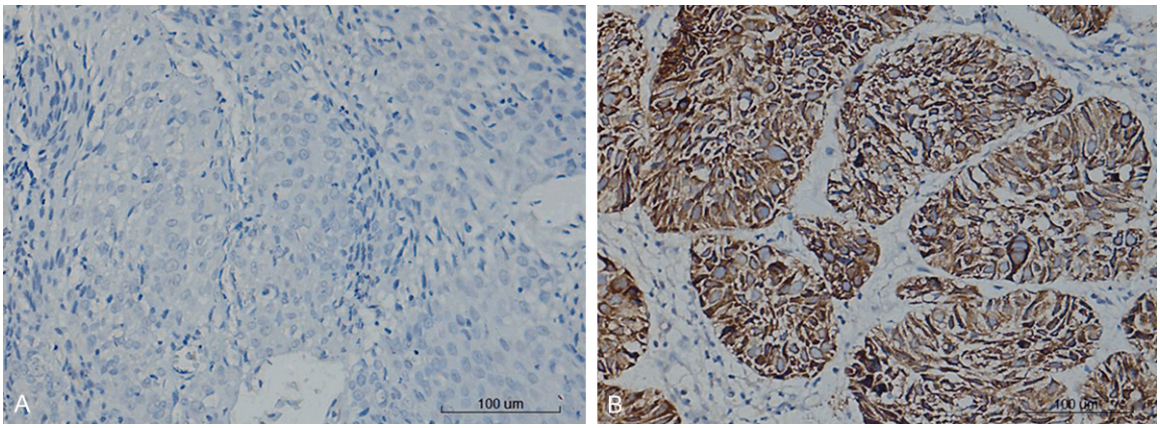

Figure 1. Immunohistochemical staining showing RASSF1 expression in ESCC tissues (×200 magnification). A: *RASSF1*-negative expression; B: *RASSF1*-positive expression.

Table 1. Analyses of *RASSF1* expression in ESCC and normal tissues

Group	Total number of cases	RASSF1		*P*
		Positive cases (%)	Negative cases (%)	
ESCC tissue	76	46 (60.5%)	30 (39.5%)	0.003
Normal tissue	20	19 (95.0%)	1 (5.0%)	

with PBS, and incubated with horseradish peroxidase-conjugated streptavidin (Shanghai Jiehao Biological technology Co., Ltd, China) for 10 min. After washing with PBS, tissue sections were developed with 3,3'-diaminobenzidine-tetrahydrochloride, stained with hematoxylin, and observed under a microscope. For the negative control, PBS was used instead of antibodies.

Immunohistochemistry results were determined according to standard procedures [15]. Positive staining of *RASSF1* was determined based on the presence of brown stains in the cytoplasm or cell membrane. Results were reviewed by two experienced pathologists in a double-blinded evaluation. Immunohistochemistry results were based primarily on the percentage of positive cells (0 points for <1%; 1 point for 1-25%; 2 points for 26-50%; 3 points for 51-75%; 4 points for >75%) and staining intensity of positive cells (0 points for negative staining; 1 point for light-yellow staining; 2 points for yellow staining; 3 points for brown staining). Points for staining intensity and percentage of positive cells were multiplied, and the specimens were classified into two groups according to their overall score: negative expression for 0-2 point and positive expression for more than 2 points.

Evaluation of tumor responses and follow-up

To evaluate tumor responses, endoscopic biopsy and chest CT were carried out 1 month after completion of radiotherapy. Follow-up CT and esophagography were done every 3 months for the first 2 years and every 6 months thereafter. Complete disappearance of the primary tumor and tumor in regional LNs upon radiology was defined as a "complete response" (CR) according to Response Evaluation Criteria in Solid Tumors v1.1.

Statistical analyses

Statistical analyses were carried out using SPSS v19.0 (IBM, Armonk, NY, USA). The χ^2 test or Fisher's exact test were used to analyze the association between *RASSF1* expression and tumor response and clinicopathologic variables. The Kaplan-Meier product-limit method was used to estimate the probabilities of overall survival (OS) and Progression-free survival (PFS), whereas the log-rank test was used to estimate any differences. OS was calculated in months from the first day of radiotherapy to the date of death from any cause or to July 2015. Patients who were alive in July 2015 were censored. *P* values <0.05 was considered statistically significant.

Results

Expression of RASSF1 protein in ESCC tissues

Positive expression of *RASSF1* in ESCC tissues was manifested as brown staining in the cyto-

Table 2. Relationship between expression of *RASSF1* protein and clinicopathologic features of ESCC

Characteristic		Number of cases	*RASSF1*		*RASSF1* positive rate (%)	χ^2	*P*
			Positive cases	Negative cases			
Sex	Male	42	25	17	59.5	0.039	0.842
	Female	34	21	13	61.8		
Age (years)	>70	38	24	14	63.2	0.220	0.639
	≤70	38	22	16	57.9		
Tumor location	Cervical	10	5	5	50.0	0.843	0.878
	Upper	20	13	7	65.0		
	Middle	39	24	15	61.5		
	Lower	7	4	3	57.1		
T stage	T1	6	4	2	66.7	2.290	0.561
	T2	35	18	17	51.4		
	T3	31	21	10	67.7		
	T4	4	3	1	75.0		
N stage	N1	54	30	24	55.5	1.929	0.165
	N0	22	16	6	72.7		
Clinical stage	I-II	26	20	6	76.9	4.447	0.035
	III-IV	50	26	24	52.0		
Grade	I	5	5	0	100.0	3.749	0.156
	II	57	34	23	59.6		
	III	14	7	7	50.0		
Tumor size (cm)	≤6	55	32	23	58.2	0.458	0.499
	>6	21	14	7	66.7		

Table 3. Relationship between treatment response and *RASSF1* expression

Expression	Clinical response		Total (n=76)	χ^2	*P*
	CR	Non-CR			
RASSF1				11.424	0.001
Positive	36	10	46		
Negative	12	18	30		

CR, complete response.

plasm or cell membranes (**Figure 1**). Statistical analyses suggested the percentage of *RASSF1*-positive cells to be 60.5% (46/76) in ESCC tissues and 95.0% (19/20) in normal tissues, and that the difference was significant (*P*=0.003) (**Table 1**).

Relationship between expression of RASSF1 protein and clinicopathologic features of ESCC

RASSF1 expression was not correlated with sex, age, tumor location, T stage, N stage, grade, or tumor size (*P*>0.05), though it displayed a significant correlation with clinical stage (*P*<0.05) (**Table 2**). Patients with a higher stage (III-IV *vs.* I-II) had significantly lower expression of *RASSF1*.

Relationship between treatment response and RASSF1 expression

A CR was noted in 48 cases (63.2%), a partial response (PR) in 15 (19.7%), stable disease (SD) in 11 (14.5%), and disease progression (PD) in 2 (2.6%). The objective response rate (RR = CR + PR) was 82.9%, and these patients were judged to be sensitive to radiation. Patients with SD or PD were judged to be insensitive to radiation. In *RASSF1*-positive and *RASSF1*-negative patients, the clinical response was CR in 36 and 12 cases, and non-CR in 10 and 18 subjects, respectively. There was a significant difference in the clinical response to radiotherapy between *RASSF1*-positive and *RASSF1*-negative groups (*P*=0.001) (**Table 3**).

Relationship between survival outcomes and RASSF1 expression

Patients were followed-up until death or for a median of 18 (range, 4-61) months in survivors. At the end of our study, 63 of 76 patients had died. Median overall survival time for all 76 patients was 18 months, with a 5-year OS of 15.1%. In the univariate analysis, survival was

Table 4. Univariate analyses of prognostic factors in ESCC

Clinical factor		Number of cases	Median survival (months)	*P*
Sex	Male	42	15.0	0.815
	Female	34	20.0	
Age (years)	>70	38	17.0	0.258
	≤70	38	17.0	
Tumor location	Cervical	10	16.0	0.533
	Upper	20	17.0	
	Middle	39	18.0	
	Lower	7	14.0	
T stage	T1	6	19.0	0.817
	T2	35	19.0	
	T3	31	14.0	
	T4	4	10.0	
N stage	N1	22	22.0	0.230
	N0	54	16.0	
Clinical stage	I-II	26	22.0	0.338
	III-IV	50	15.0	
Grade	I	5	19.0	0.929
	II	57	17.0	
	III	14	20.0	
Tumor size (cm)	≤6	55	19.0	0.374
	>6	21	13.0	
RASSF1 expression	Positive	46	23.0	0.000
	Negative	30	13.0	
Clinical response	CR	48	29.0	0.000
	PR	15	13.0	
	SD	11	10.0	
	PD	2	6.0	

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

significantly associated with tumor response (CR) and positive expression of *RASSF1* ($P<0.05$) (**Table 4**). However, other parameters showed no association with OS. Kaplan-Meier curves of *RASSF1*-positive and *RASSF1*-negative groups were drawn based on follow-up data, and 5-year OS and 5-year PFS compared between the two groups. Compared with *RASSF1*-negative patients, *RASSF1*-positive patients had higher 5-year OS and 5-year PFS, and the difference was significant ($P<0.01$) (**Figure 2A**, **2B**). Median OS was 23 months (95% confidence interval (CI), 1.243-2.449) in the *RASSF1*-positive group and 13 months (0.205-0.653) in the *RASSF1*-negative group ($P=0.007$). Five-year OS according to the clinical response showed that the CR group had better OS and PFS ($P=0.000$) (**Figure 2C**, **2D**). Multivariate analyses revealed that the tumor response (CR) and *RASSF1* expression were significant prognostic factors (**Table 5**).

Discussion

Recently, despite advances in early diagnosis and multimodal therapy, the prognosis of ESCC is quite poor. Five-year OS remains <20% [16]. Radiotherapy has been used extensively in the treatment of locally advanced esophageal cancer. However, only a small percentage of patients benefit from radiotherapy, and the outcomes vary greatly and in an unpredictable manner [17]. Pretreatment clinical parameters such as TNM classification, sex, age, and tumor differentiation cannot be used to predict the biologic behavior of patients who undergo radiotherapy [18]. Thus, identification of biomarkers that can be used to predict tumor response and treatment outcomes before radiotherapy is very important.

It has been shown that chromosome 3p21 contains a candidate tumor-suppressor gene, and it is one of the most common regions for allelic losses in ESCC [19]. Increasing evidence suggests that *RASSF1* is a novel candidate tumor-suppressor gene at 3p21, and it has been demonstrated to be a *bone fide* tumor suppressor influencing cell-cycle events, microtubule stability, apoptosis, and autophagy [20]. Recent studies have shown that expression of *RASSF1* transcripts and methylation are associated with progression and survival in ESCC [21, 22], but few studies have been conducted on the radiosensitive effect of *RASSF1* on ESCC.

In the present study, we focused on the tumor-suppressor gene *RASSF1*, and analyzed the association of *RASSF1* expression with radiosensitivity and prognosis in ESSC. Studies have revealed increased expression of *RASSF1* in normal human tissues [13]. Investigations based on immunohistochemistry have suggested that the prevalence of positive expression of *RASSF1* decreases progressively in normal and ESCC tissues. In the present study, loss of

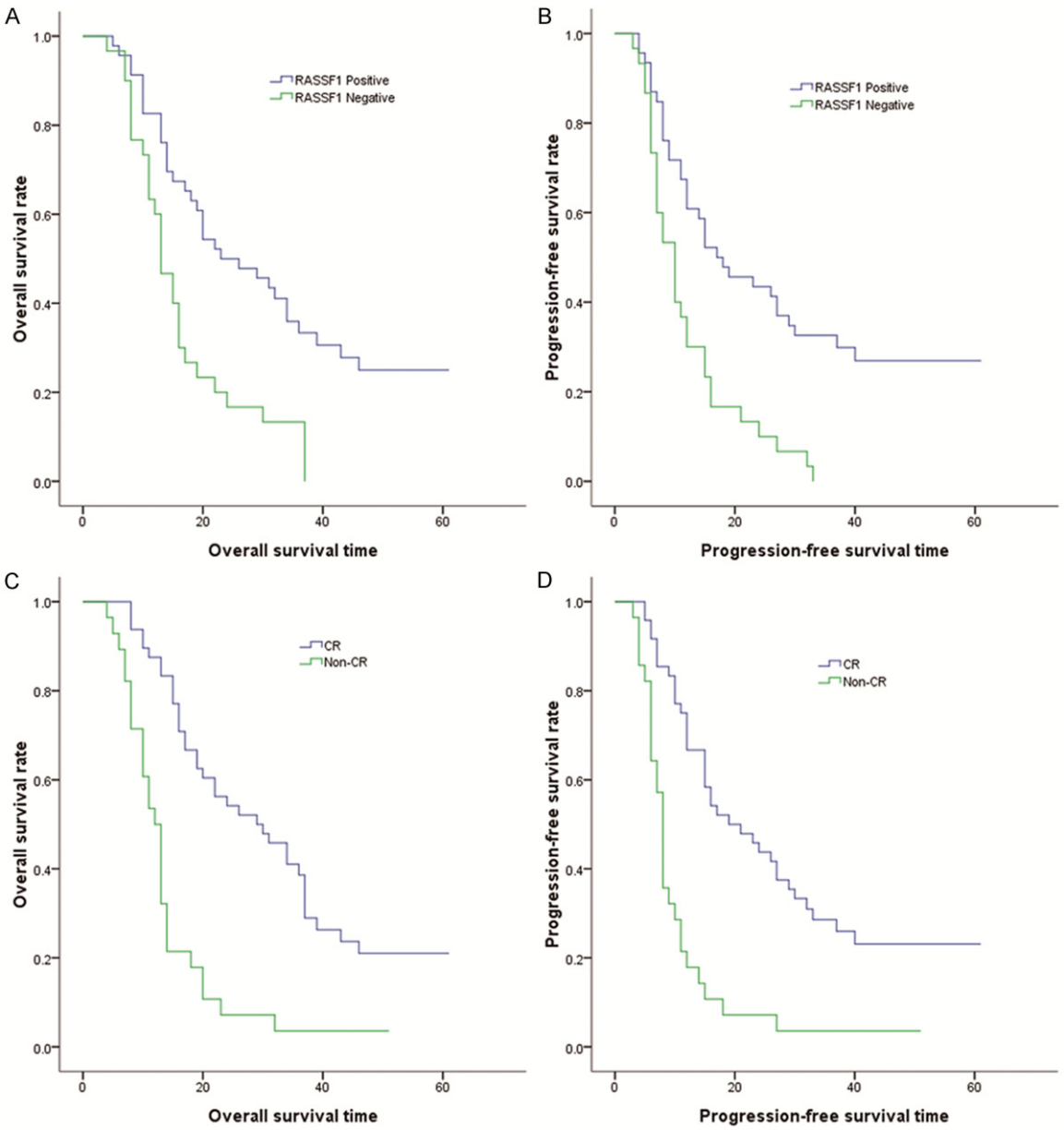

Figure 2. Kaplan-Meier survival curves for ESCC patients treated by RT according to RASSF1 expression and CR. A. Correlation between OS and RASSF1 expression. B. Correlation between PFS and RASSF1 expression. C. Correlation between OS and CR. D. Correlation between PFS and CR.

Table 5. Multivariate analyses of prognostic factors in ESCC

Clinical factor	*P*	Hazard ratio	95% CI
RASSF1	0.000	3.387	1.964-5.841
Clinical response	0.000	4.741	2.683-8.378

expression of *RASSF1* protein was found to be more obvious in ESCC tissues than in the non-tumor tissues, suggesting that *RASSF1* may act as a tumor-suppressor gene in esophageal cancer. After correlation analyses of protein expression and clinicopathologic factors, positive expression of *RASSF1* was found to be associated with clinical stage because the percentage of patients with *RASSF1*-positive expression was higher in stage I-II than in stage III-IV, but it was not correlated with sex, age, tumor location, T stage, N stage, grade, or tumor size. We noted a significant difference in response to radiotherapy in ESCC, and *RASSF1*-positive expression led to high sensitivity to

radiotherapy. Therefore, *RASSF1* expression in ESCC can help to predict the effectiveness of radiotherapy.

Multivariate analyses of 10 variables, including clinicopathologic and *RASSF1* factors, showed that treatment response and *RASSF1* expression were significant prognostic factors for OS. Compared with *RASSF1*-negative patients, *RASSF1*-positive cases had higher 5-year OS and 5-year PFS. Thus, *RASSF1* expression could be used as a parameter for prediction of the survival of ESCC patients before radiotherapy.

Our study had two main limitations. First, *RASSF1* protein was detected using IHC staining, the results of which can be influenced by the detection method used and subjective evaluation. Second, our study was retrospective; prospective studies are needed to confirm our findings.

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Disclosure of conflict of interest

None.

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